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Calcium as a Limiting Factor in the Distribution of the Amphipod *Gammarus pseudolimnaeus*

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CALCIUM AS A LIMITING FACTOR IN THE DISTRIBUTION OF THE
AMPHIPOD *GAMMARUS PSEUDOLIMNAEUS*

A Thesis

Presented to

The Faculty of the Department of Biology

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

by

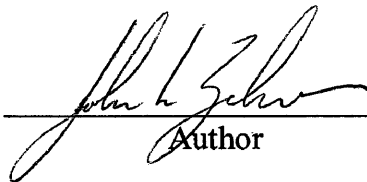
John Kinsley Zehmer

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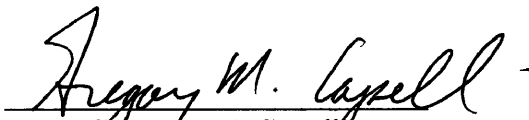
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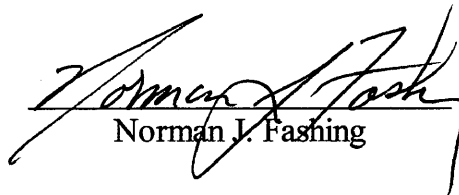
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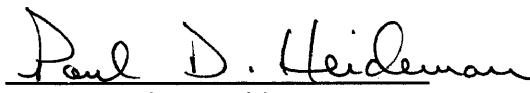
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Approved, May 1998


Gregory M. Capell


Norman J. Fashing


Paul D. Heideman

for my parents

thank you for your love and support

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Abstract

The amphipod *Gammarus pseudolimnaeus* is abundant throughout the majority of the length of two small streams on the coastal plain of Virginia, but is absent from their upper reaches. The streams exhibit a gradient of dissolved materials and pH, with conductivity ranging from 12 to 250 μmhos , and pH ranging from under 6 to 7, from source to lake. General osmoregulatory failure during intermolt does not appear to be limiting the distribution of this amphipod; percent body water content, a general gauge of osmotic stress, was not found to be greater in amphipods closer to the distribution limit, nor does it increase in amphipods displaced above the distribution limit. In the laboratory, males and females maintained in water from above the distribution limit experienced greater mortality than those in water from below the distribution limit. Mortality was closely associated with time of molt. Amphipods in water from above the distribution limit generally lived 1-2 days post-molt while those in water from below the distribution limit lived, on average, 8-10 days post-molt. The addition of Ca^{2+} to water from above the distribution limit increased post-molt survival to levels observed in those maintained in water from below the distribution limit; raising pH had no effect on post-molt mortality. Insufficient ambient calcium appears to be a limiting factor in the distribution of *G. pseudolimnaeus* in these two streams.

CALCIUM AS A LIMITING FACTOR IN THE DISTRIBUTION OF THE
AMPHIPOD *GAMMARUS PSEUDOLIMNAEUS*

Introduction

Freshwater amphipods are widely distributed among diverse habitats. With densities that may exceed 10,000 / m² (Pennak 1978), they often form an important link between primary and secondary production (Hanson and Waters 1974). However, relatively few studies have examined the environmental conditions affecting or limiting their distribution. Most are either purely descriptive of ranges or suggest connections between distribution and chemical factors (e.g. alkalinity, pH, Ca²⁺, Mg²⁺, and conductivity) without determining if those factors are actually limiting.

One of the most commonly mentioned factors is calcium. The distribution of at least one species from most orders of freshwater animals has been associated with this ion (Macan 1961). Calcium is important for intracellular processes and, in many aquatic animals, for maintaining an impermeable membrane (Rasmussen and Bjerregaard 1995; McDonald 1983; Macan 1961). In some crustaceans it is also important for haemocyanin oxygen-binding properties (Mangum 1983; Taylor and Spicer 1986) and for hardening the exoskeleton (Stevenson 1985; Hilton et al. 1984). The distributions of the Gammarid amphipods *Gammarus minus* Say, *G. fossarum* Koch, *G. roeseli* Gervais, and *G. pseudolimnaeus* Bousfield have all been correlated with calcium (Glazier and Gooch 1987; Schrimpf and Foeckler 1985; Mahon 1997). However, Sutcliffe and Carrick (1973) suggested that bicarbonate,

with which calcium is often associated, was more important than calcium to the distribution of *G. pulex* (L.)

Freshwater invertebrate diversity generally declines with lower pH (Schell and Kerekes 1989; Okland and Okland 1986); crustaceans appear to be especially acid intolerant (Havas and Hutchinson 1983; Okland and Okland 1986). Among amphipods, the following lower pH limits have been reported: *G. pulex*, 5.7 (Otto and Svensson 1983; Sutcliffe and Carrick 1973), though one survey described this species at pH 4.5 (Pinkster 1972); *G. lacustris* Sars, 6.0 (Okland and Okland 1986); *Hyalella azteca* Saussure, 5.6 (Grapentine and Rosenberg 1992; Stephenson and Mackie 1986).

Associations between chemical factors and species distribution can be inferred from survey data, but the covariance of many factors and the interaction among factors make interpretation difficult. For example, although pH is commonly cited as limiting in many organisms, the threshold where deleterious effects manifest in any given species is influenced by the chemical and physical background. For example, increased dissolved humic substances reduced the mortality of *G. pulex* in acidic water (Hargeby and Petersen 1988; Hargeby 1990). Increased sodium concentration ameliorated the toxic effects of low pH on *G. fossarum* (Meinel et al. 1985), and calcium level may be especially important in relation to pH in many animals (Okland and Okland 1985). Therefore, manipulative experiments are important for interpretation of survey results.

The amphipod *Gammarus pseudolimnaeus*¹ is found in two small streams, on the coastal plain of Virginia. Both streams exhibit a strong chemical gradient, including calcium and pH, from source to mouth. *G. pseudolimnaeus* is abundant throughout most of the length of these streams but has a sharp distribution limit in the upper reaches of both. Mahon (1997), using field displacement experiments, found significantly higher mortality rates above the distribution limit compared with those below. Neither predation nor interspecific competition appeared to be factors in the mortality. Based on the mortality pattern, Mahon (1997) speculated that death occurred at molt.

The experiments described here address potential factors limiting the distribution of the amphipod *G. pseudolimnaeus*. I describe field and laboratory experiments to test the hypothesis that general osmoregulatory failure prevents range expansion. In two laboratory experiments I tested for differences in mortality between males and females. Finally, chemical manipulation experiments in the laboratory tested the hypothesis that Ca^{2+} and/or pH are limiting factors.

¹ The specimens closely match published descriptions of *Gammarus pseudolimnaeus*, a species previously known from the Great Lakes and upper Mississippi drainages. This finding has been confirmed by Dr. John Holsinger of Old Dominion University, who suggests using the designation *Gammarus* (near) *pseudolimnaeus*. For convenience, however, the organism will hereafter be referred to in this document as simply *Gammarus pseudolimnaeus*.

Animals and Study Area

The two study streams, designated Berkeley and Pogonia (see Mahon 1997), are located on the coastal plain of Virginia on the campus of The College of William and Mary within a forest preserve. Both arise as springs and flow about 900m (Berkeley) and 700m (Pogonia) before confluencing with a small lake. The streams have a very low flow over mostly firm, sand substrate, varying throughout from 0.3 to 10m in width with depths generally less than 10cm. Dissolved O₂ is near saturation for most of their lengths, but the streams exhibit a strong gradient of dissolved materials, with conductivity ranging from 12 to 250 μ mhos from source to lake.

G. pseudolimnaeus is abundant throughout most of the length of both streams; however, in the upper reaches of both it declines from abundant to absent over a distance of only a few meters. This area in both streams will be referred to as the distribution limit (DL). The DL in both Pogonia and Berkeley is very low in dissolved materials with conductivities of approximately 49 μ mhos and 28 μ mhos, respectively (Figure 1). The exact location of this area and the associated chemical conditions tend to remain constant (Greg Capelli, personal communication). Sites were established 50m above and 15m below the DL in Berkeley and 25m above and 25m below the DL in Pogonia for use or reference in the experiments described below. The upper and lower sites will be referred to as ADL (above the DL) and BDL (below the DL), respectively.

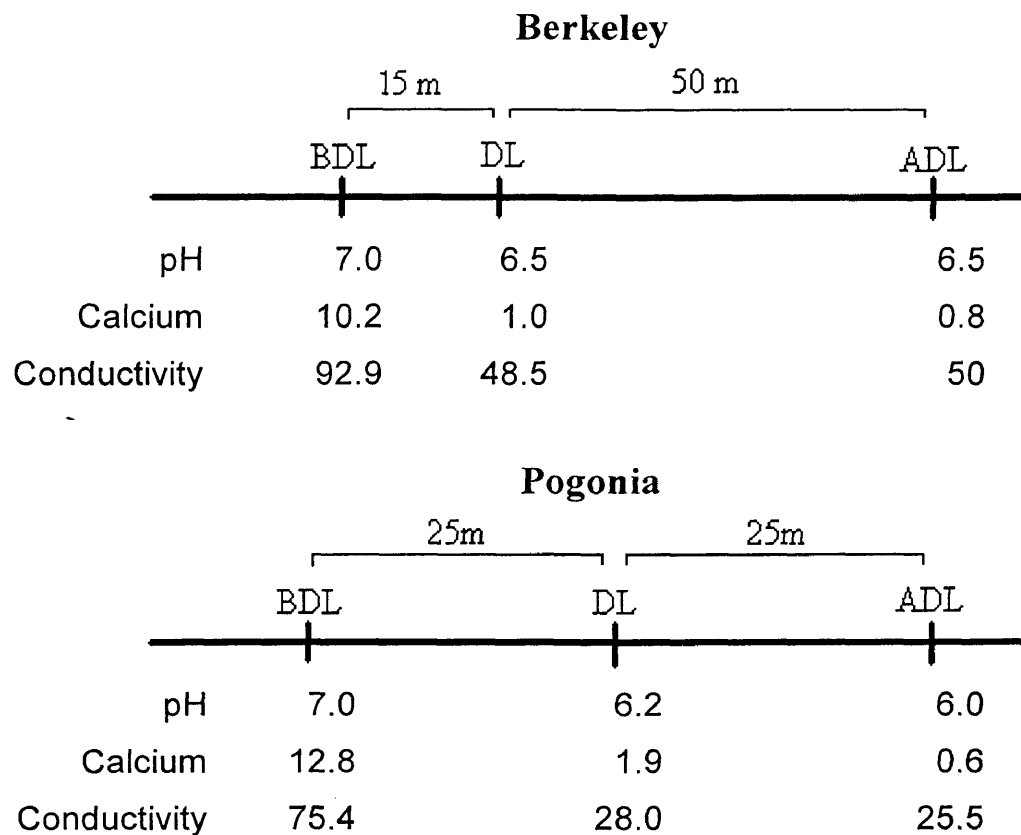


Figure 1. Schematic of Berkeley and Pogonia in the vicinity of the distribution limit (DL). DL represents the distribution limit of the amphipod *Gammarus pseudolimnaeus*. BDL and ADL are sites below and above the distribution limit, respectively, used in the experiments described in the text. Mean values are reported. Calcium is reported as mg Ca^{2+} / l and Conductivities are in μmhos .

Materials and Methods

General Field and Laboratory Methods

Amphipods were collected by placing leaf litter into a sieve and retrieving the captured individuals. All individuals used for experimentation were adults ranging from 8 to 14 mm in length. In the laboratory, the light cycle was kept at 14:10 (L:D) and the temperature was maintained at approximately 20 °C. All amphipods collected for laboratory experiments were kept together in the laboratory in a 55 by 30cm tank with water from the collection site for three days prior to use. Chemical determinations were made as follows: calcium tests were performed using the Hach Chemical Company's digital titration method; conductivity measurements were made using a Fischer Traceable conductivity meter; determination of pH was made using a Fischer Accumet Model 5 pH meter.

Water Balance

Crustaceans living in dilute media compensate for osmotic influx by increasing the volume of urine production at the antennal gland (Mantel and Farmer 1983). Body water content has been used as an indicator of osmotic stress since, below a threshold ambient osmolarity or below a threshold pH increased osmotic influx results in increased body water content (Sutcliffe 1971; Hargeby and Petersen 1988; Hargeby 1990). I hypothesized that, if general osmotic failure

accounted for the absence of *G. pseudolimnaeus* above the DL, a non-lethal trend involving increasing water content might occur in an upstream direction toward the DL. I collected 30 unsexed amphipods from each of 3 sites in Berkeley: 350 m below the DL (175 μmhos), 150 meters below the DL (120 μmhos), and 15 meters below the DL (93 μmhos). The amphipods were brought to the laboratory, randomly paired to provide sufficient mass for accurate measurement, and blotted dry with absorbent paper. Pairs were weighed together to the nearest 0.1mg, dried for 24 hours at 50 $^{\circ}\text{C}$, and weighed again to determine percent water content.

I also tested, in a field displacement experiment, whether body water content increases in animals maintained in the lethal area of the stream, above the DL. In the field 60 unsexed amphipods were collected from Berkeley, 100m below the DL (109 μmhos) and randomly distributed between two flow through cages (20cm x 14cm x 5cm deep). One cage was placed at site BDL and the other at site ADL. Both were retrieved after 5 days since preliminary laboratory experiments indicated that amphipods in ADL water began to experience mortality by day five. Surviving amphipods were treated and measured as above. Additionally, 60 amphipods were collected as above and maintained in aquaria for five days in the laboratory in either ADL or BDL water. Survivors were treated and measured as above.

Male and Female Mortality

There may be sex differences in tolerance of environmental stressors. This experiment tested for differential survival of males and females maintained in water from site ADL and site BDL. Precopula pairs, which allow rapid determination of

sex, were collected from Berkeley, 100 m below the DL. Individuals were separated by sex in the field and brought to the laboratory. Twenty females and 20 males were each assigned randomly to one of two treatment groups: water from the ADL site or from the BDL site. Amphipods were maintained individually without food in glass jars 11 cm tall by 6 cm in diameter with 100ml of water. For 14 days, water in each jar was changed daily, molts and deaths were recorded, and exuviae were removed. The experiment was performed twice.

Chemical Manipulation

The following laboratory experiment tested the hypotheses that low pH or insufficient calcium reduces post-molt survival of *G. pseudolimnaeus*. Control treatments consisted of unaltered ADL and BDL water. In one experimental treatment, pH of ADL water was raised, through the addition of NaHCO_3 , to a level empirically observed to support amphipods a short distance below the DL. In another treatment, calcium concentration was similarly increased through the addition of CaCl_2 . The addition of NaHCO_3 or CaCl_2 increased the osmolarity of the water, thereby reducing the osmotic gradient between amphipod and medium; this could have reduced mortality, confounding the results. This was controlled for, in the procedural control, through the addition of NaCl to ADL water sufficient to match or exceed the total number of ions introduced in either of the two experimental treatments. The addition of CaCl_2 or NaCl did not significantly alter the pH of the ADL water in any of the experiments.

Two replicate experiments were performed using amphipods and water from Berkeley. In the NaHCO_3 treatments, pH was raised to 6.9 ± 0.2 and 6.7 ± 0.2 , respectively. In the CaCl_2 treatments the calcium concentration was raised to $10.6 \pm .9 \text{ mg Ca}^{2+}$ and $10.6 \pm 1.1 \text{ mg Ca}^{2+}$, respectively. One experiment was performed using amphipods and water from Pogonia. The pH in the NaHCO_3 treatment was raised to 6.6 ± 0.1 ; the calcium concentration in the CaCl_2 treatment was raised to $10.6 \pm .8 \text{ mg Ca}^{2+}$.

In each experiment 100 unsexed amphipods were collected 100m below the DL. Twenty amphipods were randomly assigned to each of the five treatment groups. Amphipods were maintained individually in jars with 100ml water. An 8mm disc of beech leaf, previously incubated in stream water for at least three days, was replaced in each jar, every two days as a food source. The water was changed daily, molts and deaths were recorded, and exuviae were removed. Amphipods that molted within 14 days from the start of the experiment were followed until their death or until a total of 14 days had elapsed from their time of molt.

Results

Water balance

Treatment variances were homogenous (Levene's test, $p = .99$). ANOVA and multiple comparisons using Tukey's test were applied to the data. The treatments differ ($p = .002$). Amphipods collected from 350m and 150m below the Berkeley DL did not differ significantly in water content ($71.5 \pm 0.13\%$, $72.4 \pm 0.15\%$, respectively; $p = .45$; Table 1). Water content of amphipods collected 15m below the DL ($69.7 \pm 0.12\%$) was significantly *lower* than that of amphipods collected 150m below the DL ($p=.002$), and those collected 350m below the DL ($p=.04$).

In the field displacement experiment 24 individuals survived in the ADL treatment and 28 in the BDL treatment. The surviving individuals were randomly paired for accurate weighing resulting in sample sizes of 12 and 14, respectively. Treatments variances were homogenous (Levene's test, $p=.53$). An unpaired t-test with standard error adjusted for unequal sample sizes was applied to the data. Percent water content of amphipods maintained for 5 days at the ADL site ($72.4 \pm 0.74\%$) did not differ significantly ($p = .66$) from those at the BDL site ($72.7 \pm 0.08\%$).

In the laboratory water balance experiment 14 individuals survived in the ADL treatment and 26 in the BDL treatment, providing sample sizes of 7 and 13,

Field Survey			
	Meters Downstream from DL		
	15	150	350
% Water Content	$69.7 \pm 0.12\%$ (15)	$72.4 \pm 0.15\%$ (15)	$71.5 \pm 0.13\%$ (15)
Field Experiment			
	Treatment		
	ADL	BDL	
% Water Content	$72.4 \pm 0.74\%$ (12)	$72.6 \pm 0.08\%$ (14)	
Laboratory Experiment			
	Treatment		
	ADL	BDL	
% Water Content	72.1 ± 0.17 (7)	73.0 ± 0.14 (13)	

Table 1. Results from the three water balance experiments. The mean percent water content with the standard error is presented for each treatment with the sample size in parentheses. In the field survey amphipods collected from 15m below the distribution limit (DL) had significantly lower percent water content ($p = .02$) than the other two treatments, which did not differ. The percent water content of amphipods in the ADL and BDL treatments did not significantly differ in either the field or laboratory experiments.

respectively. The treatment variances were homogenous (Levene's test, $p=.47$) and an adjusted t-test was applied as above. Amphipods maintained in ADL water ($72.1 \pm 0.17\%$) did not differ ($p=.25$) from those in BDL water ($73.0 \pm 0.14\%$).

Male and Female Mortality

The survival curves were analyzed by proportional model regression analysis using StatView Survival Tools. For comparisons among curves, α was adjusted to .008 for an experimentwise error rate of .05. In both experiments ADL females experienced higher mortality than BDL females ($p<.0001$ for both). Mortality of females and males maintained in the same water, ADL or BDL, did not differ significantly in either experiment (see Figures 2,3 for p values). However, in the first experiment ADL males tended to survive longer than ADL females ($p = .06$) and males in BDL water tended to survive for less time than BDL females ($p = .06$). Females in ADL water tended to have higher mortality than males in BDL water ($p=.024;.016$); ADL females had significantly higher mortality than BDL females ($p=.0001;<.0001$; figures 2,3). Males in ADL water tended to have greater mortality than males in BDL water ($p = .12$; $p=.011$).

In both experiments mortality appeared to be closely associated with time of molt in both sexes. In the two experiments 48 females and 17 males molted. In both experiments no amphipods maintained in ADL water survived for longer than 2 days post-molt and most lived less than or equal to 1 day post-molt. Most amphipods maintained in BDL water, regardless of sex, lived longer than 5 days post-molt.

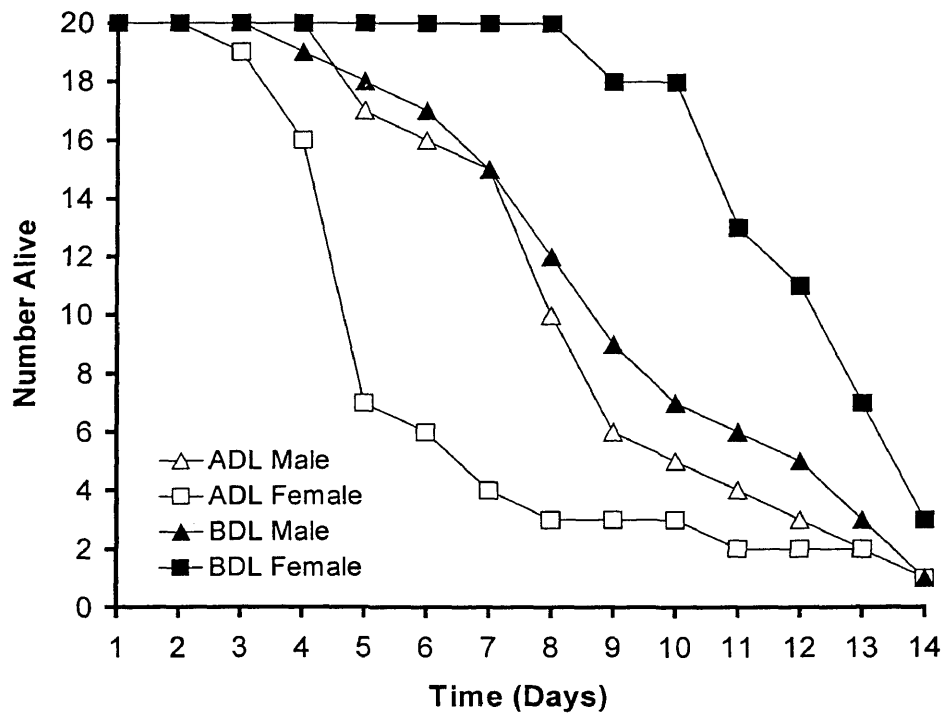


Figure 2. Survival curves for male and female amphipods from Berkeley maintained in ADL and BDL water in experiment 1. Proportional hazards model regression analysis with α adjusted to .008 gave the following results: The curves differ ($p < .0001$). ADL males do not differ significantly from ADL females ($p = .06$). BDL males and females do not differ ($p = .06$). BDL females survive longer than ADL females ($p < .0001$) but do not differ from ADL males ($p = .03$). BDL males do not differ from ADL females ($p = .02$) or from ADL males ($p = .7$).

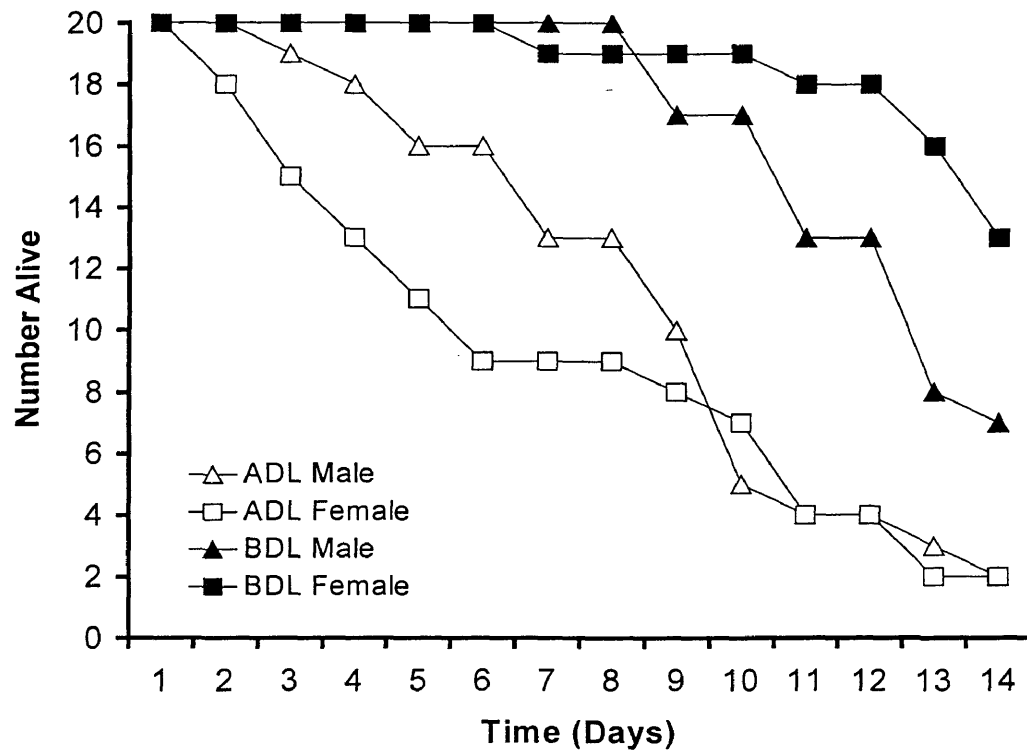


Figure 3. Survival curves for male and female amphipods from Berkeley maintained in ADL and BDL water in experiment 2. Proportional hazards model regression analysis gave the following results: The curves differ ($p < .0001$). ADL males did not differ significantly from ADL females ($p = .86$). BDL males and females did not differ ($p = .082$). BDL males survived longer than ADL males ($p = .011$) and females ($p = .016$). BDL females survived longer than ADL males ($p < .0001$) and females ($p = .0001$).

Chemical manipulation

Consistent with Mahon's (1997) suggestion and the observations reported above, differential mortality rates in the chemical manipulation experiment resulted from differential post-molt survival in the various treatments. In all treatments mortality occurred in some individuals prior to molting but did not differ significantly among treatments (proportional model regression analysis; first Berkeley experiment, $p=.56$; second Berkeley experiment, $p=.87$; Pogonia experiment, $p=.47$).

The results for post-molt survival of the two Berkeley experiments were qualitatively and quantitatively similar and the data were combined in a single analysis. The $\log_{10}(X+1)$ transformed data (Zar 1996) had homogenous variance (Levene's test; $p=.79$). The transformed data were analyzed in a 2-way ANOVA with treatment as 1 factor with 5 levels and experiment as the second factor with 2 levels. The effect of treatment was significant ($p<.0001$). There was neither an effect of experiment ($p=.22$), nor a significant interaction ($p=.51$) and so the data were collapsed across the second factor for multiple comparisons using Tukey's test.

Amphipods in the BDL treatment survived an average of 9.6 days post-molt, significantly longer than amphipods in the ADL treatment, which survived an average of 1.9 days ($p<.0001$, Figure 4). The ADL and the NaCl treatments did not differ significantly ($p>.5$). Amphipods in the CaCl_2 treatment survived an average of 8.9 days post-molt, longer than the ADL treatment ($p<.0001$), and not significantly

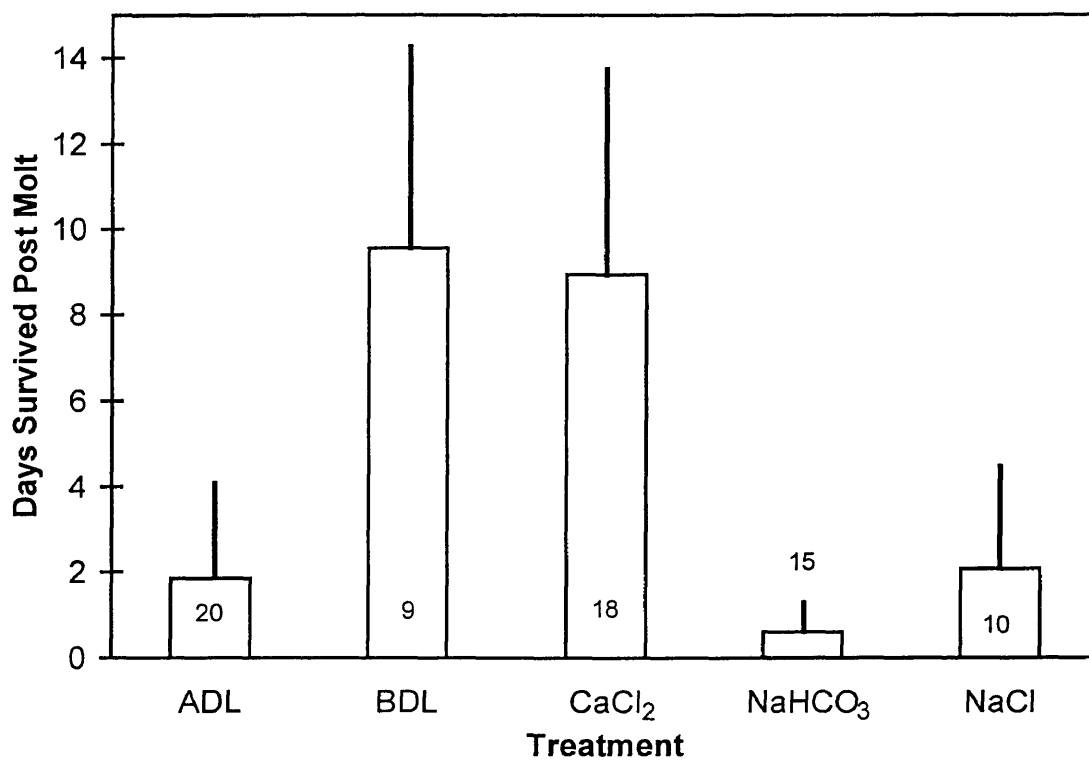


Figure 4. Results from the 2 chemical manipulation experiments with amphipods and water from Berkeley. Error presented as standard deviations; numbers in bars are sample sizes. The treatments differ (2-way ANOVA, $p < .0001$) but there was no effect of experiment ($p = .22$) nor an interaction ($p = .51$). Multiple comparisons were made with Tukey's test after collapsing data across experiments. ADL and NaCl do not differ ($p > .5$) and NaHCO₃ does not differ from ADL ($p = .087$). BDL and CaCl₂ do not differ ($p > .5$) and both are significantly greater than ADL ($p < .0001$ for both).

different from the BDL group ($p > .5$). The NaHCO_3 group survived an average of 0.6 days post-molt, which was not significantly different from the ADL group ($p = .087$).

In the Pogonia experiment the $\log_{10}(X+1)$ transformed data had homogenous variance ($p = .24$). The transformed data were analyzed with a 1-way ANOVA which was significant ($p < .0001$). Multiple comparisons were performed using Tukey's test.

Results were similar to those for Berkeley. Amphipods in the BDL treatment survived an average of 8.3 days post-molt, significantly longer than amphipods in the ADL treatment, which survived an average of 1 day post-molt ($p < .0001$; Figure 5). The ADL and NaCl treatments did not differ significantly ($p > .5$). Amphipods in the CaCl_2 treatment survived an average of 10.7 days post-molt, longer than the ADL treatment ($p < .0001$), and not significantly different from the BDL group ($p = .33$). The NaHCO_3 group survived an average of 1 day post-molt, which was not significantly different from the ADL group ($p > .5$).

Although potential food in the form of beech leaf detritus was provided for all experiments, amphipods consumed very little.

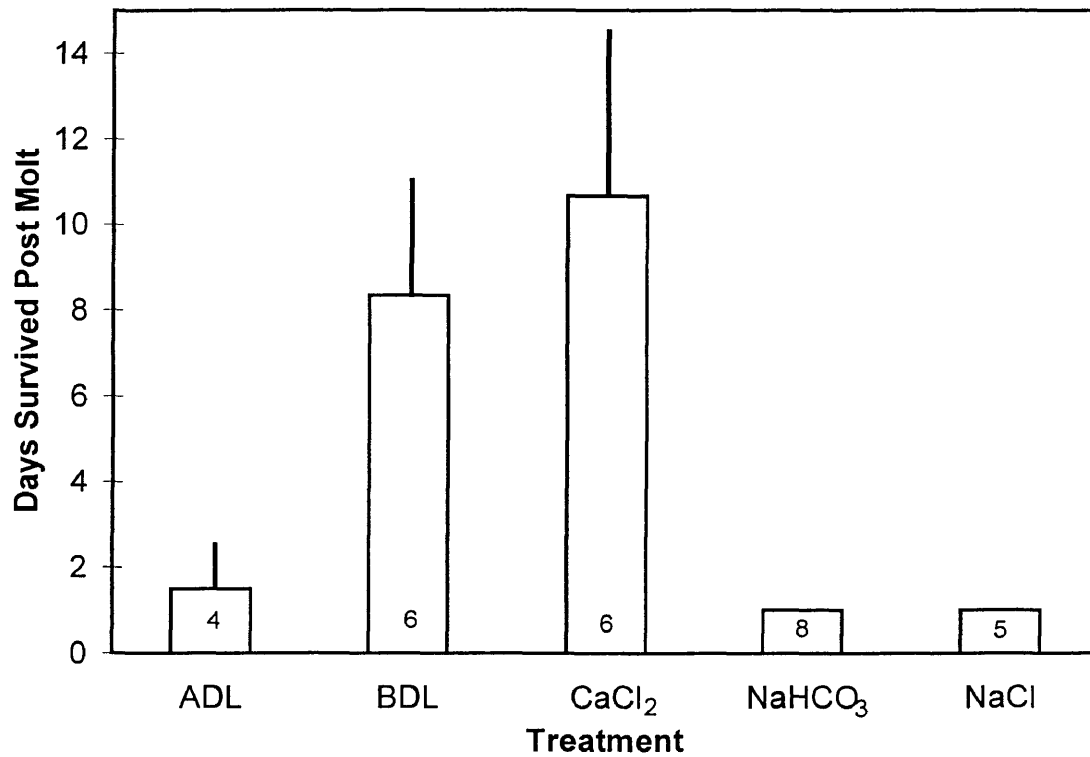


Figure 5. Results from the chemical manipulation experiment with amphipods and water from Pogonia. The treatments differ (1-way ANOVA, $p < .0001$). ADL, NaCl, and NaHCO₃ do not differ ($p > .5$). BDL and CaCl₂ do not differ ($p = .33$) and both are significantly greater than ADL ($p < .0001$; $p < .0001$). All amphipods in the NaHCO₃ and NaCl treatment groups died after 1 day post-molt, resulting in an estimated variance of 0.

Discussion

Amphipods displaced above the DL in flow-through cages, in both Berkeley or Pogonia, experienced higher mortality than those in cages below the DL (Mahon 1997). Many biotic or abiotic factors could potentially account for this. However, the 2mm mesh on the flow-through cages used in Mahon's work excluded all likely predators and competitors. The change from abundance to absence in the two streams occurs over only a few meters. Furthermore, the chemical conditions found at the distribution limits of the two streams are similar in pH and calcium concentrations (Figure 1). This suggests some aspect of water chemistry as the limiting factor.

The water at the DL in Pogonia and Berkeley is very low in dissolved materials. For crustaceans living in dilute media, concentration gradients drive ion loss and osmotic influx which is typically balanced by enhanced ion uptake and an overall reduction in integument permeability. However, while the permeability of the gills, gut, and antennal glands is, in most cases, generally reduced in freshwater species with respect to those structures in marine species, the need to respire, absorb nutrients, and excrete wastes limits this reduction (Mantel and Farmer 1983). Body water content has been used as an indicator of osmotic stress since below a threshold ambient osmolarity, or in depressed pH, increased osmotic influx

results in increased body water content (Sutcliffe 1971; Hargeby and Petersen 1988; Hargeby 1990). For example, when the brackish amphipod *G. duebeni* is displaced into dilute media its body water content increases from 78% to 80%. *G. pulex* maintained in a stream with a pH around 7 had a body water content of 73% while those maintained in streams with lower pH's had body water contents up to 80% (Hargeby 1990).

Amphipods maintained in ADL water in the field and in the laboratory showed only slight and insignificant increases in body water content (0.2% and 0.9%, respectively). Furthermore, in the field survey, the *lower* body water content of amphipods collected at site BDL (15m below the DL), compared to the other 2 sites further downstream of the distribution limit, is inconsistent with the hypothesis that amphipods nearer the distribution limit are progressively more osmotically stressed. Therefore, a general osmoregulatory problem does not appear to prevent intermolt animals from living further upstream.

There may be sex differences in tolerance of environmental stressors. In the male and female mortality experiment, no significant differences were found between males and females in either ADL or BDL water. However, the p-values were very close to .05 (figure 2,3) and a real difference may exist. In the BDL water, males tended to have higher mortality than females. This may represent a lower threshold for laboratory stress in males, a difference in life span between the sexes, or some selection bias related to choosing males from precopula pairs. Males collected from precopula pairs varied widely in size and, presumably, age.

Energy expended in mate guarding could lower males' tolerance for stressors, but females may also have additional energy needs associated with reproduction.

The lower survival of females in ADL water, however, is probably a result of their higher molting rates. In the 2 experiments 48 females molted, compared to 17 males. This is probably a direct result of their collection from precopula pairs. While the males could have been at almost any stage in the molt cycle, females in precopula pairs are typically at late stage C, shortly before molt-stage (Wright 1980). Analysis of mortality in relation to molt in the 2 water types revealed a striking pattern. While male and female amphipods in BDL water survived many days post-molt, those in ADL water rarely lived longer than 1 day post-molt. Unfortunately, I tracked the amphipods for only the 14 days of the experiment and not until the death of all the molted animals. The resulting data censor the survival of 16 of the amphipods in BDL treatments and 1 in the ADL treatments. Nonetheless, the resulting conservative estimate of post-molt survival clearly demonstrates that survival post-molt for males and females is acutely impacted by the water in which the amphipods are maintained. Most of the difference in mortality between the ADL and BDL treatments is due to differential mortality at molt. These findings confirm Mahon's (1997) speculation that death above the DL is largely molt related.

I hypothesized that low pH or low calcium concentration of water above the distribution limit in these streams is limiting to *G. pseudolimnaeus* at the time of molt. I tested this hypothesis through the addition of CaCl_2 and NaHCO_3 to ADL

water, in separate treatments, to raise the calcium concentration and pH, respectively, to levels found just below the DL, where amphipods were always present. The rise in pH associated with the addition of NaHCO_3 did not decrease mortality. However, the effect of calcium was clear. Increasing ADL calcium concentration to 10.6 mg Ca^{2+} resulted in a reduction in post-molt mortality to levels found in BDL water in the 3 experiments. No increase in survival over the ADL group occurred upon addition of an equal total number of Na^+ and Cl^- ions. Thus, the increase in survival observed in the CaCl_2 treatment could not have been due to the rise in ambient osmolarity associated with the addition of Ca^+ and Cl^- ions. Ambient calcium concentration may impact post-molt survival through an epidermal membrane association or as a source for maintaining internal calcium concentration.

In crustaceans, permeability to water can increase by as much as two times intermolt levels during ecdysis (Lockwood and Inman 1973). Increased water uptake results in swelling which aids in the expansion of the new exoskeleton (Mantel and Farmer 1983). However, with the loss of the barrier of the exoskeleton, this probably represents the period of greatest osmotic stress (Hargeby and Petersen 1988). Calcium is important in many animals for maintaining a relatively impermeable epithelial membrane. In fish gills, calcium is bound to negatively charged proteins on the surface of the epithelium at tight junctions, stabilizing the membrane, and thus lowering permeability (Hunn 1985; McDonald 1983). Gill permeability of fish typically increases with decreasing ambient calcium

concentrations (Potts and Flemming 1970; McWilliams 1983; Ogasawara and Hirano 1984; Pic and Maetz 1981). Furthermore, in some fish low pH may displace calcium from its bound sites on the epithelium at cell tight junctions leading to increased permeability. This effect is minimized in high calcium concentrations (McWilliams 1983; McDonald 1983). Ionoregulatory failure, probably through this process, is the primary mechanism of mortality in moderately low pH (McDonald et al. 1980).

However, the role of calcium in the permeability of the crustacean membrane is less clear and subject to interspecific variation. The apparent water permeability of the crab *Cherax destructor* and the crayfish *Astacus astacus* show little relation to calcium concentration and the crab *Carcinus maenas* increases its permeability with increased ambient calcium (Rasmussen and Bjerregaard 1995). However, the crab *Cancer magister* experiences a reduction in permeability to Na^+ ions in high calcium media (Robinson and Potts 1979). Unlike in fish, calcium concentration had little effect on sodium loss in the cladocerans *Daphnia magna* and *Acantholeberis curvirostris* in low pH (Potts and Fryer 1979) or on the mortality of the amphipod *G. fossarum* in low pH (Meinel et al. 1985).

Ecdysis is also characterized by the loss of the majority of body calcium, 96% in *G. pulex*, both into solution and in the form of the exuviae (Wright 1980). Immediately after molt the calcium balance is shifted to rapid uptake, though the rate of this process is dependent on ambient calcium concentration (Wright 1980) and pH (Malley 1980). Therefore, *G. pseudolimnaeus* may survive above the DL

during intermolt through retention of body calcium, but calcium concentration above the DL may be too low for efficient uptake at molt. This would prevent the calcification of the new exoskeleton, but this alone in a culture jar, in the absence of predators, would be unlikely to result in immediate mortality. However, if calcium loss during ecdysis, without efficient uptake, briefly drops body calcium below concentrations necessary for general internal functions, an inability to rapidly reacquire calcium from the environment might result in mortality.

In the Berkeley and Pogonia chemical manipulation experiments, amphipods maintained in BDL water or in ADL water with additional calcium lived an average of about 9 days post-molt. Therefore, the mechanism of mortality in these groups is probably not molt-related. However, this is probably a higher mortality than found in the field below the DL. Conditions may have been more stressful in some respect in the laboratory than would be the case in the field. It would be instructive to follow amphipods in BDL water in the laboratory to determine if any survive to a second molt. Although periodic checks revealed no hypoxia in the culture jars and water was changed daily to prevent waste build up, a less static environment may be important for amphipod survival.

Insufficient nutrition may also have played a part in laboratory-related stress. Beech are abundant in the forest surrounding the study streams and the stream water-incubated beech leaves were included as an energy source for the chemical manipulation experiments. However, throughout the experiment, few amphipods ate visible portions of the leaf discs. Fungal colonies are important in the

palatability of leaf litter to amphipods (Barlocher and Kendrick 1973) and the three day incubation period may not have been sufficient for establishment of important fungal colonies. It is also possible that beech leaves may, in some other way, be unacceptable as a food source. Osmoregulation requires substantial energetic input (Nery and Santos 1993) and energetic demands in ADL water may exceed those of BDL water. This could be especially important to amphipods molting later in the experiments when energy reserves between the groups of animals would possibly be most different. However, there is probably no role for food as a calcium source as uptake through the gut from food was not a significant source of ions in four species of talitrid amphipod (Morritt 1989).

Conclusions and Future Directions

Fresh waters with both low conductivities and low calcium concentrations are common throughout the Appalachian mountains and in much of the northern portion of the United States and into Canada. An understanding of the role of calcium as a limiting factor for other crustacean species in these and other areas would be valuable to both biogeography and to biomonitoring efforts. All crustaceans lose a significant fraction of their body calcium at molt in the form of the exuviae and substantial loss from the haemolymph and other compartments has been reported in the crabs *Callinectes* (Hecht 1914) and *Uca* (Kleinholz 1941), the crayfish *Austropotamobius* (Greenaway 1974), and the amphipod *Gammarus* (Wright 1980). Therefore, low calcium concentration may be a factor in the distribution of many other crustaceans in areas depauperate in calcareous materials.

No systematic survey of the distribution of *G. pseudolimnaeus* in relation to water chemistry has been published. Such a study would be valuable in determining the applicability of these results to *G. pseudolimnaeus* distribution in other areas. Furthermore, more manipulative experiments such as those described here need to be performed on other species. However, an understanding of the mechanism of mortality for *G. pseudolimnaeus* in low calcium waters would provide a stronger basis for predicting the importance of calcium in other situations. The connection between molt and mortality does not resolve the role calcium plays in promoting post-molt survival; there are many possible explanations as explored in the discussion. Furthermore, these results should not be interpreted as an absolute lower calcium limit

for *G. pseudolimnaeus*. The calcium concentration at the distribution limit in Berkeley is 1mg Ca²⁺/l and at the limit in Pogonia is 1.9mg Ca²⁺/l. In the chemical manipulation experiments calcium concentration was raised to around 10mg Ca²⁺/l, much higher than that found at the distribution limits. Therefore we cannot conclude based on this evidence that calcium alone is sufficient to explain the observed distribution limits. The difference in the calcium concentration at the distribution limits in the two streams may be explained by an interaction with other chemical factors that moderate the lower physiological limit for *G. pseudolimnaeus*.

Therefore, more study is needed with experiments like those described here as well as experiments designed to address the physiological mechanisms involved in calcium/molt-related mortality. I give here three examples of experiments that could begin to explore this physiological question: The osmolarity of ADL water could be raised through the addition of an inert organic molecule like sucrose and the effect on post-molt mortality determined. Decreased mortality would suggest that post-molt mortality in low calcium media is somehow related to osmoregulation. Tracer studies using labeled water (see Lockwood and Inman 1973) could be used to determine if *G. pseudolimnaeus*'s permeability to water and ions changes with ambient calcium concentrations. This experiment would be especially valuable if performed shortly after molt. Positive results would suggest the importance of calcium in maintaining the impermeability of the membrane. Finally, amphipods could be allowed to molt in BDL water in which a calcium channel antagonist had been introduced. Reduced survival under these conditions would suggest that the role of calcium is internal, and that membrane-bound calcium is not an important factor.

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Vita

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